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Nucleic Acid Sequence Coding for Enzyme Delta-12-Desaturase and Originating from *Frigilariopsis Cylindrus*, Associated Polypeptide, and Use of Both

Specification

The present invention relates to a nucleic acid sequence coded for the delta-12-desaturase (Δ12-desaturase) enzyme, to the associated polypeptide and to uses of the nucleic acid itself as well as of the associated polypeptide.

Enzymes from the Δ12-desaturases catalyze an important step towards synthesizing linoleic acid which is essential for humans and which serves as an important structural element of the cellular membrane and which, by the eicosanoids developed by it, controls many life-essential processes in the organism. Moreover, linoleic acid which man cannot produce himself due to his lacking Δ12-desaturase enzyme, is the basis for the synthesis of further essential fatty acids such as, for instance, arachidonic acid (AA, eicosatetraenic acid ETA), eicosapentaenic acid (EPA) and docosahexaenic acid (DHA).

Fig. 1 depicts a general diagram of the biosynthesis in eukaryotes of polyunsaturated fatty acids (PUFAs) and of the associated enzymes. The conversion of stearic acid (18 carbons: 0 double bonds) to oleic acid (18:1, Δ9) is catalyzed by a Δ9-desaturase. Oleic acid is converted to linoleic acid (18:2, Δ9, 12; abbreviated LA) by a 12Δ-desaturase, and linoleic acid is in turn converted by a Δ6-desaturase to γ-linolenic acid (18:3, Δ9,12,15, abbreviated GLA), or to α-linolenic acid (18:3, Δ9,12,15, abbreviated ALA) by a Δ15-desaturase. The elongation of the fatty acids is catalyzed by

elongases, by which dihomo-γ-linolenic acid (20:3, Δ8,11,15; abbreviated DGLA) is formed from γ-linolenic acid. Dihomo-γ-linolenic acid is in turn converted to arachidonic acid (20:4; Δ5,8,11,15; abbreviated ARA) by a Δ5-desaturase. Arachidonic acid is a direct precursor of the physiologically effective eicosanoids such as, for instance, prostaglandine, thromboxane, leukotriene.

The family of the PUFA's or omega fatty acids (ω fatty acids, with ω - counting from the methyl-end), to which the names fatty acids belong, generally affect the internal surfaces of the blood and lymph vessels, the function of the white blood cells, blood coagulation and infection and immune reactions. Diets deficient in these essential fatty acids may lead to disturbances of the corresponding physiological processes. The German Gesellschaft fuer Ernaehrung (German Society for Nutrition) is recommending a daily supply of at least 12.5 g of linoleic acid which is only taken in with a very balanced diet. It is for this reason that food additives are being offered and basic food stuffs, e.g. bread are enriched with basic fatty acids. The measures are to assist in an improved control of the cardio-vascular diseases.

Since vertebrates cannot insert double bonds behind position 9, at Δ-counting from the carboxy-end, into fatty acids, unsaturated fatty acids such as LA and ALA, or the Δ12- and Δ15-desaturase enzymes which catalyze them, are essential nutrients the supply of which must be chiefly provided from vegetable or animal sources. Most PUFA's in humans and animals derive either from food such as fish and predetermined plants (olive, oenothera, borage) or they are developed from essential fatty acids supplied by food by conversion with desaturases and elongases. For this reason, the organisms in which they occur naturally are of great commercial interest. Research programs of the pharmaceutical and food industry include a constant search for new sources and for appropriate micro-organisms in particular. Beyond the direct production of PUFA's from such organisms,

knowledge of the genes of the PUFAS biosynthesis effective in them is of particular interest because of present-day possibilities of gene-technology. By a deliberate, functional expression of the genes in such host plants as soy beans or corn (maize), commercial production of PUFA's can be achieved in such better accessible systems. For that reason, there is a need for genes for desaturases and elongases of the PUFAS biosynthesis as well as for the production of PUFA's by economical methods aided by those genes.

The function of desaturases in the metabolism of fat is sufficiently known. Hence, a long list of publications exists which relates to the provision of such enzymes, their amino acid sequences and their coding genes. For instance, WO9411516 relating to "Genes for microsomal delta-12 fatty acid desaturases and related enzymes from plants" (1994) contains an extensive disclosure of present knowledge of the metabolism of fatty acid. It describes Δ12-desaturases from plants, e.g. soy beans, oil seed *brassica species*, *arabidopsis thaliana* and corn (maize). It discloses the use of "gene-chimerata" for the transformation of plants, i.e. the manipulation of the host genome by insertion of foreign genes by means of vectors for an increased production of essential fatty acids. A Δ12-desaturase coded by a nucleic acid sequence of the common hazelnut is known from EP 0794250. WO0020602 "Delta 6 and delta 12 desaturases and modified fatty acid biosynthesis and products produced therefrom" (2000) claims Δ6- and Δ12-desaturases, nucleic acid sequences coding for such proteins, DNA-structures containing such genes and micro-organisms which express increased quantities of such desaturases. Preferably, the desaturases are isolations from the oil-containing mushroom *mortierella alpina*. DE 100 44 468 "New nucleic acid encoding delta-6-desaturase, useful for producing ciliates and plants that overproduce unsaturated fatty acids, derived from tetrahymena" (2001) describes a γ-linolenic acid derived from linoleic acid catalyzing Δ6-desaturase derived from the ciliate *tetrahymena thermophila*. This is a thermophile ciliate with a maximum activity at elevated temperatures.

However, the commercial production of essential fatty acids from natural sources entails several disadvantages. Their quality as well as their quantity varies and in some cases they are heterogenous compounds which require purification steps. By comparison, it has been found that the yield with some micro-organisms by comparison with higher plants is markedly higher. For that reason, the production of essential fatty acids by micro-organisms offers a promising alternative to other PUFAS sources. The fatty acid spectrum of many micro-organisms often is rather simple compared to higher organisms. This offers significant advantages in cleaning. Moreover, the fermentative production does not depend upon external factors such as weather, food availability, etc. Also, PUFA's produced in this manner are substantially free of contaminations which may be traced, for instance, to environmental contamination. A further advantage is that PUFA's derived from fermentative processes are not subject to variations in their availability, in contrast to those derived from natural sources.

Furthermore, all of the currently known organisms for producing fatty acids cited in the publications referred to above, as well as all other currently known organisms for the production of fatty acids come from zones of temperate climates. Therefore, for producing essential fatty acids, all suitable organisms require process conditions which correspond to their natural climatic ambient conditions and, more particularly, markedly increased process temperatures. Hence, heat supply devices, in particular complex breeder reactors, are required for the production.

From the publication I "Zahllose Geheimnisse der Natur" (Countless Secrets of Nature) by K. Eske (*vide* BioLOG, 3rd Edition, February 2000, pp. 2 and 3 downloadable at http://www.Bioregio.org/biolog-3.pdf, status 4 June 2002) it is known to isolate enzymes adapted to coldness from bacteria which occur in deep sea regions. Aside from the advantage that enzymes adapted to coldness require no raise temperatures for their expression, the deep-sea organisms in question are potentially abundantly available since 80% of the

water covering the earth are of a temperature below 5 °C. The proportion of PUFA's which prevent congealing of the cellular membrane is especially high in micro-organisms from cold regions. The temperature of the maximum activity of these organisms and their functional components lies significantly below that of organisms from temperate and tropical areas. By using micro-organisms adapted to coldness, even in a gene-technologically modified form, production at low temperatures and much lower energy consumption is decisively more economical than conventional production methods with organisms not adapted to coldness.

In light of these important findings, it is an object of the present invention to provide an organism adapted to coldness which has a nucleic acid sequence which at cold processing temperatures encodes a delta-12-desaturase for a coldness-adapted enzyme, for an economical production of essential fatty acids based upon a delta-12-desaturase enzyme. The object is accomplished by claim 1. Advantageous developments and applications which relate to the polypeptide associated with the inventive nucleic acid sequence are the subject of subclaims and alternative independent claims.

The solution in accordance with the invention satisfy the requirements placed upon the invention in an optimal manner. On the one hand, the organism having the claimed nucleic acid.sequence has a delta-12-desaturase enzyme which is particularly suited for the catalysis of an important step for the production of essential fatty acids, and on the other hand, the organism originates in the arctic sea so that the enzyme is one which is adapted to coldness and does not require added heat for its activity. Knowledge of such a gene is of elementary importance, since it encodes for a coldness-adapted Δ12-desaturase enzyme which is of particular interest in connection with the cultivation of plants in temperate and colder regions. The microbial synthesis of fatty acid with the rapidly growing diatom with its coldness-adapted enzyme provides high yields at low temperatures.

As confirmed by performed sequence analyses, the nucleic acid claimed by the invention codes for a Δ12-desaturase enzyme. The present developed methods of automatic sequencing of nucleic acid segments have made it possible within reasonable intervals of time purposefully to isolate from promising organisms genes of desired characteristics. Extensive public data bases containing sequences associated with defined known functions serve to verify the results of a tedious search. A number of known working steps serves to make available the processes basic material required for the sequencing:

1) Isolation and Cultivation of the *fragilariopsis cylindrus* Organism.

Isolation: During an antarctic journey in the Weddell Sea o board the German research vessel "Polarstern", the diatom was isolated from sea ice. Its type classification was performed in a simple manner by typing the structure of the shell (see, in this connection, the publication II by Medlin & Priddle: "Polar marine diatoms", 2nd edition,, British Antarctic Society, Cambridge, 1990).

Cultivation: The diatom was placed in a flowing medium 2x f/2 enriched by nutrient salts at 0 °C at light of 10μmol photons $m^{-2}s^{-1}$ (see, in this connection publication III by Guillard & Ryther: "Studies of marine plankton diatoms, I. Cyclotella nana (Husted) and detonula confervacea (Cleve)", 1962, Can. J. Microbiol. 8, 229:239). To increase the expression of the genes responsible for the coldness adaptation of the type, the algae were cooled down to -2 °C halfway through their exponential growth phase. This corresponds to the freezing point of sea water. After five days the messenger RNA (mRNA) were isolated from the algae. All messenger RNA represent the active genes, including those responsible for the coldness adjustment.

2) Isolation of the mRNA.

The complete RNA was isolated by means of the RNAeasy Plant Mini Kit (Qiagen Co.). From about 100 µg of RNA it was possible, by means of the Oligotex mRNA Midi Kit (Qiagen Co.), to isolate about 800 ng RNA for the cDNA synthesis.

3.) Fabrication and Screening of a cDNA bank.

The cDNA bank was produced with a SMART cDNA library construction kit (Clontech Co.) on the basis of the mRNA:

A) For this purpose, the first cDNA strand was synthesized from the mRNA by means of oligo nucleoside and CDS III/3' primers;

B) Thereafter, double strand synthesis was realized in an Eppendorf Thermocycler by means of LD-PCR (long distance polymerase chain reaction), using the following program:

C) 1. 5 min of denaturation at 95 °C followed by 20 cycles of 6 min at 68 °C and 2 min at 95 °C;
2. After SFiI digestion (with a restriction enzyme from *streptomyces fimbriatus*) of the cDNA, it was fractionated according to size in CHROMA Spin-400 columns so that only cDNA of a length > 400 bp (base pairs) were used for cloning;
3. These cDNA's were ligated overnight at 16 °C in TriplEX2 vectors which could be received by λ-phages. The titer of this cDNA bank was at 2.7 x 10^9 pfu/ml (plaque forming units);
4. Blue-white screening with IPTG (isopropyl-b-D-thiogalactoside) and X-gal (X-galactoside)_ displayed a recombination efficiency of 70%;
5. This cDNA bank was screened by means of a digoxygenine marked Δ12-desaturase derived from *phaeodacttylum tricornutum*;
6. Hybridization took place overnight at 50 °C;
7. Stringency washing took place in 2 x SSC / .5 x SDS.

4. Sequence Analysis.

Positive phage plaques were sequenced from their 5'-end with λ-primers by the Qiagen Sequencing Service. The sequences were checked with the BLAST option for their homologies with existing sequences at the gene bank of the NCBI (National Center for Biotechnology Information) (25 February 2002). The results of the comparison displayed score values between 50 and 80. One of every 20 positive phage plaques could be positively identified as Δ12-desaturase.

Fig. 2 depicts the result of a phylogenetic analysis. As may be seen, the desaturase from *fragilariopsis cylindrus* groups with significant bootstrap support (97% or 99%) with other Δ12-desaturases. Hence, it can be said that with a probability bordering on certainty that the coldness adapted enzyme coded in accordance with the claimed nucleic acid sequence from the diatom *fragilariopsis cylindricus* is a Δ12-desaturase.